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LC-MS CHARACTERIZATION AND CELL-BINDING PROPERTIES OF CHELATE MODIFIED SOMATROPIN

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INTRODUCTION

Somatropin, a recombinant protein containing 191 amino acids, is derived from the endogenous human growth hormone, somatotropin [1]. This protein is clinically used in children and adults with inadequate endogenous growth hormone to stimulate a normal bone and muscle growth. In addition, somatropin is currently being investigated for the diagnosis and radiotherapy of certain hormonal cancers. In some of these cancers, over-expression of the human growth hormone receptor (hGHR) is described.
The modification of the protein with a chelating agent like NOTA (1,4,7-triazacyclononane-1,4,7-triacetic acid) allows the inclusion of metals coupled to the protein. The NOTA unit is selectively introduced on a lysine side chain.
As site-specific labelling is necessary to avoid active region interactions, characterization of the chelate-modified somatropin is indispensable. Therefore, we have applied an enzymatic digestion procedure using trypsin, chymotrypsin and a combination of both enzymes. The resulting peptides were then monitored using HPLC-MS^n, allowing the investigation of the exact position of amino acid modifications. Moreover, the intact protein, without enzymatic degradation, was analysed on a protein HPLC column using UV detection for quantification and ESI-MS/MS for characterization. The cell-binding functionality of the characterized NOTA-somatropin was also measured.

EXPERIMENTAL

After NOTA-labelling of somatropin [2], the cysteine residues were first reduced and alkylated using DL-dithiothreitol (DTT) and iodoacetamide, respectively. The solution was then desalted, using a PD-10 desalting column. Subsequently, an aliquot of the solution was incubated with trypsin (4 hours, 37°C), chymotrypsin (24 hours, 37°C) and a combination of both enzymes (24 hours, 37°C). The resulting peptides were separated using a Vydac Everest C_{18} column (250 mm x 2.1 mm i.d., 5 µm particle size, 300 Å) in an oven set at 45°C, with a mobile phase consisting of A) 0.1% w/v formic acid in water, and B) 0.1% w/v formic acid in acetonitrile. A linear gradient was employed and the flow rate was set at 0.2 mL/min.
The intact somatropin (1 mg/ml) was analysed using a Vydac C_4 column (250 mm x 4.6 mm i.d., 5 µm particle size, 300 Å) in an oven set at 35°C, with a mobile phase consisting of A) 50 mM ammonium bicarbonate buffer, pH 7.5, and B) n-propanol. An isocratic elution was employed and the flow rate was set at 0.5 mL/min.
The cell binding experiments were performed using LigandTracer®. Therefore, the NOTA-modified somatropin was labelled with gallium-68 and incubated with the HepG2 cell line.

RESULTS and DISCUSSION

1. Characterization of NOTA-modified somatropin

Based upon the HPLC-MS^n results of the digested somatropin, the chelating molecule is mainly bound to a specific lysine amino acid that is located away from the receptor binding site and which has a particularly low pKa value [2]. These findings are confirmed by bio-informatics.
The use of an enzyme mixture of trypsin and chymotrypsin is suitable for localisation of modified lysine amino acids. In that way, an enhanced information efficiency is obtained.

Table 1: Digested NOTA-modified somatropin

Enzyme	General lysine coverage	Specific lysine amino acid
Trypsin	100 %	11 %
Chymotrypsin	67 %	18 %
Trypsin and chymotrypsin combination	89 %	17 %

2. Characterization of intact NOTA-modified somatropin

The developed method for LC-MS characterization of intact somatropin was suitable to identify the protein (Figure 1).
After quantification of the NOTA-modified somatropin sample, about 95% of somatropin was NOTA-modified.

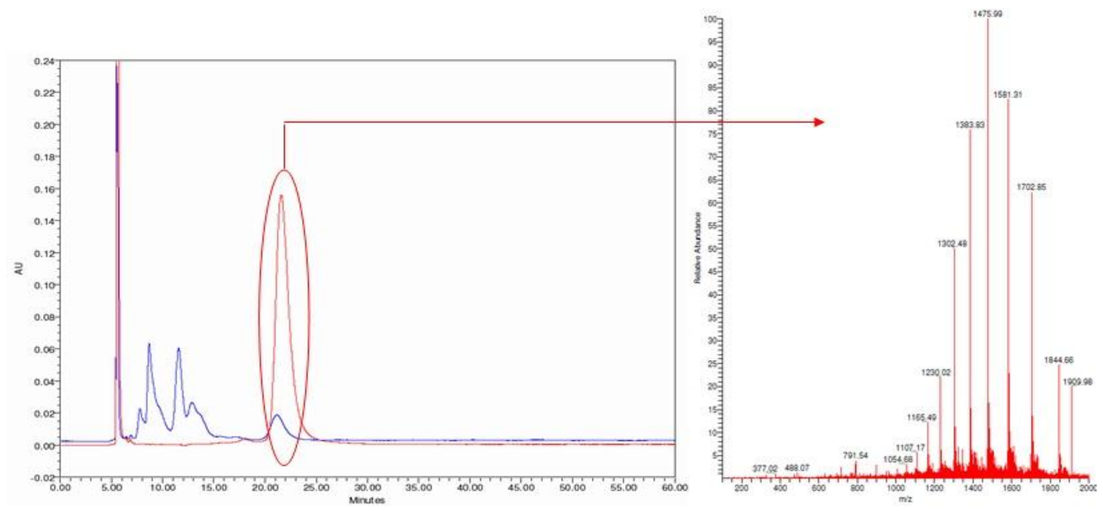

Figure 1: HPLC chromatogram (left) of somatropin (red) and NOTA-modified somatropin (blue), together with the mass spectrum (right) of somatropin.

3. Cell-binding functionality of NOTA-somatropin

Initial cell-binding results of gallium-68 labelled NOTA-somatropin indicate that the modified protein binds to the HepG2 cells.

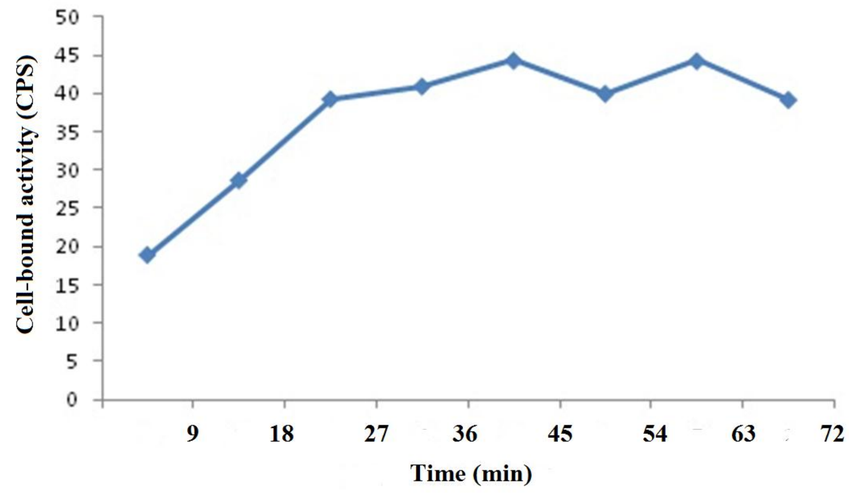

Figure 2: Cell-binding results of NOTA-modified somatropin

CONCLUSIONS

The use of a mixture of trypsin and chymotrypsin is most suitable for the peptide-mapping characterization of chelate modified somatropin. The use of this enzyme combination gives an enhanced information efficiency. Moreover, the developed method for LC-MS characterization of intact somatropin is suitable to identify the protein.
The initial cell-binding functionality test of the NOTA-modified somatropin indicate that the protein binds to the HepG2 cells.

REFERENCES

[1] European Pharmacopoeia 6.0, 01/2008:0951.
[2] Patent application GB1006285.9